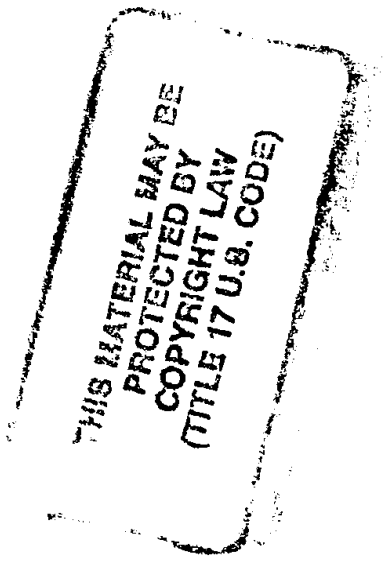
Preparing Transgenic Animals with a Simplified Method of Morula Aggregation Using ES Cells

Weihong Kong, PhD.
Pyapalli Usha Rani, MS.
Yunhua Bao, MS, and
Jaspal Singh Khillan, PhD

The authors describe an ES cell technique that resolves the problems of time, expense, and inconsistency often encountered in the production of transgenic mice via DNA microinjection.



The authors are affiliated with The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Please send reprint requests to Khillan at the above address.

Transgenic animals present a powerful system for the study of gene regulation during development and differentiation in an intact animal system[1]. Researchers have prepared several lines of transgenic animals to investigate the effect of gene mutations, as well as to develop animal models for human genetic disorders[2]. Micromanipulation of the preimplanted embryo inserts a gene of interest into the animal genome, which is then transmitted to the subsequent generations in a normal Mendelian fashion.

Transgenic animals are generally prepared by microinjection of DNA into the pronuclei of newly fertilized embryos[3,4], which integrates DNA into the host genome. Although microinjection of DNA is quite efficient, it has several limitations: for example, the technique requires highly skilled workers and involves expensive equipment. Additionally, the efficiency of DNA integration into embryos can vary from 0.1% to over 30%. The DNA is usually randomly integrated as a tandemly arranged multimeric unit[1,5], which results in wide variations in gene expression. In some integrants, the gene is completely shut off by the flanking host genomic sequences, so the animal may not exhibit any phenotype. Consequently, there may be unnecessary losses of time, effort, and valuable resources. A procedure that allows one to predetermine the site of integration for gene expression, and allows for a low gene-copy number (thus limiting variability), can greatly expedite the outcome of transgenic experiments.

Pleuripotent embryonic stem (ES) cells[7,8], commonly used to create gene knockout mice via homologous recombination[9], may be a convenient alternative technique for transgenic animal preparation. Preparing transgenic animals via ES cells offers several advantages. For example, the cells can be pre-selected for site of integration, low gene-copy number, and high expression of the gene. However, the potential of ES cells to create transgenic mice for random DNA integrants has not been fully exploited because of specialized conditions required to maintain these cells[10]. ES cells also have certain limitations—for example, only gene constructs with promoters that are active in ES cells can be used, and the expression of gene constructs with promoters that are turned off in ES cells cannot be predicted. Using a gene cassette containing a gene for a selectable marker driven by a promoter, that is active in ES cells, along with the gene of interest with its own promoter can overcome such limitations. The neomycin resistance (*neo*) gene, driven by the promoter of Herpes Simplex virus thymidine kinase (*HSV TK*) gene or phosphoglycerokinase (*PGK*) gene, has been used extensively in ES cells. After the introduction of DNA, cells can be selected with G418[4,10] (an analog of neomycin), and the clones for high expression and low gene-copy number may be identified to prepare chimeric animals.

In general, chimeric animals are prepared by microinjecting ES cells into blastocysts[11]. As with DNA microinjection, this procedure also requires expensive equipment and specialized skills. An alternative method is to prepare chimeric animals by aggregation of ES cells with preimplantation embryos[12-14].

We describe here a simple method to prepare chimeric animals that display a high ES cell contribution[15]. The procedure

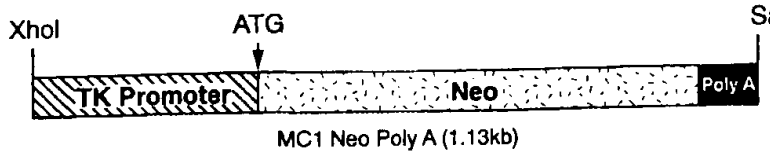


FIGURE 1. 1.13 kb XhoI-SalI neomycin resistance gene construct which contains Herpes simplex virus thymidine kinase promoter (TK) and polyadenylation signal. The DNA contains about 106 bp of HSV TK promoter (bar with cross lines), 792 bp *neo* gene (hatched bar), followed by poly A signal (black bar). The DNA used for microinjection was purified using Qiagen Kit (Qiagen Inc.) and dissolved in Tris EDTA buffer pH 7.2.

does not involve any major equipment or specialized skills, and therefore can be used by ordinary laboratories to prepare genetically modified animals. In this report, we demonstrate the preparation of transgenic animals by aggregation of morula[3] and ES cells. The approach may be used to prepare transgenic animals for any gene for which a gene cassette is designed to contain a gene for a selectable marker. The procedure may also be applicable to other species for which researchers have developed ES cell lines[16-18].

Materials and Methods

Preparation of DNA

An approximately 1.13 kb XhoI-SalI DNA fragment, which contains *HSV TK neo* gene with poly A signal (Fig. 1), was excised from pMC1neo poly A plasmid[19] (purchased from Stratagene Inc., La Jolla, CA). The DNA contains about 106 bp of HSV TK promoter and 792 bp of modified *neo* gene. The DNA was purified using Qiagen Kit (Qiagen Inc., Valencia, CA) and dissolved in Tris EDTA buffer pH 7.2.

Electroporation of DNA into ES cells

Normal R1 ES cells were cultured over primary embryonic fibroblast (PEF) feeders in 60 mm petri dishes using ES cell-medium supplemented with 1000 IU/ml of lymphocyte inhibitory factor (LIF; Esgro, Gibco-BRL Pharmaceuticals, Rockville, MD) according to procedures described by Robertson[10].

Confluent cultures were treated with 1 ml of 0.25% Trypsin EDTA (Gibco-BRL Pharmaceuticals) for 3-5 minutes at 37°C. The cells were washed two times with ES medium, followed by washing with phosphate buffered saline (PBS), and then resuspended in PBS. Approximately 25 µg DNA were electroporated into 5 x 10^6 cells at 250 V and 500 µF using a BioRad Pulsar I electroporator[20]. The cells were transferred to six 100 mm petri dishes with PEF feeder cells. After 48 hours, the cells were treated with 500 µg/ml G418. The medium was changed every day to remove non-viable cells and cell debris.

Independent clones were collected after 8-10 days and propagated in 24-well plates. After 2-3 days the cells were harvested, and an aliquot of the cells was transferred to fresh 24-well plates without feeder cells, and the remaining cells were frozen in liquid nitrogen. When the cells became confluent, total DNA was isolated and analyzed by polymerase chain reaction (PCR) using primers neo-2 (5′-TGC GCC ATT GAA CAA GAT GG-3′) and neo-3 (5′-GAG CAA GGT GAG ATG ACA GG-3′) that amplify a 330 bp internal fragment of the neomycin gene. The PCR conditions were 94°C (30 seconds), 56°C (30 seconds), and 72°C (1 minute) for 30 cycles in PEC 9600 thermocycler. Positive clones were further analyzed by Southern analysis using p32-labeled probe specific for *neo* gene.

Isolation of Embryos

Three- to four-week-old C57BL/6 female mice, purchased from the National Institutes of Health (NIH) FCRF (Frederick, MD), were superovulated with 5 IU of pregnant mare serum gonadotropin (PMSG; Gestyl; Professional Compounding Centers of America Inc., Houston, TX) followed 48 hours later by 5 IU of human chorionic gonadotropin (HCG; Sigma Chemical Co., St. Louis, MO). Although not sexually mature, the young females exhibited a high yield of embryos in response to hormone treatment. The females were mated with males from the same strain, and pregnant females were sacrificed 2.5 days after mating. Eight to 16 cell embryos were collected by flushing the uterine horns with either M16 or Brinster's medium (Gibco-BRL Pharmaceuticals, Life Technologies, Grand Island, NY). The embryos were stored in the same medium under paraffin oil in a CO_2 incubator.

Preparation of ES Cells for Morula Aggregation

ES cells were prepared for morula aggregation as described earlier[15]. The cells were harvested by trypsin treatment and dispersed as single-cell suspensions by pipetting several times. The cells were pelleted by centrifugation at 1,000 rpm for five minutes at 4°C, washed one time with ES medium, and resuspended in 10 ml of the same medium in a 50 ml Falcon tube. The tube stood for ten minutes to allow feeder cells to settle down, after which the top 2 ml of the cell suspension were collected. The cells were centrifuged for two minutes at 1,000 rpm at room temperature in a microfuge centrifuge, followed by washing two times in medium A (M16 or Brinster's medium with 10 mg/ml final

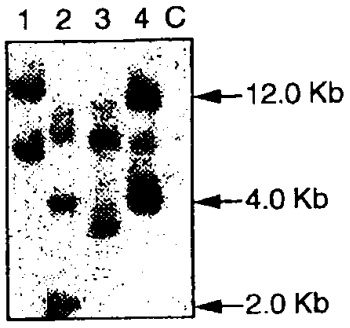


FIGURE 2. Southern blot analysis of ES cell clones resistant to neomycin is shown. Four independent clones that were resistant to 500 µg/ml G418 were digested with BamHI. The DNA was probed with a p32-labeled *neo* gene. Clone 1 showed only two bands of almost equal intensity, indicating single-copy integration of the *neo* gene.

TABLE 1. Results of preparing animals using morula aggregation.

# of embryos	# of foster mothers	# of pups			Chimeric pups
	Total	Pregnant	Born	Survived	
140	19	11	48	34*	14 (41%)**

* Fourteen pups were either cannibalized or died due to starvation.
** Numbers in parenthesis represent percentage of chimeras from the total number of surviving pups.

concentration of Bovine Serum Albumin, BSA; Pentex, Miles Inc., Kankakee, IL)[15]. The cells were resuspended in the same medium at a concentration of 1.5-2.0 x 10^4 cells/ml.

Preparation of Zona-Free Embryos

About 30-50 healthy morulas were transferred into a drop of 100 µl acid Tyrode solution (137 mM NaCl; 2.7 mM KCl; 0.5 mM $MgCl_2$ $6H_2O$; 5.6 mM glucose; 1.6 mM $CaCl_2$ $2H_2O$; 0.4% polyvinylpyrrolidine pH 2.5) in a 60 mm petri dish. Immediately after dissolution of zona pellucida, 1 ml of medium A was added to neutralize the Tyrode solution followed by washing of embryos in 3-5 ml of fresh medium A[15].

Aggregation of Embryos and ES Cells

About 100 µl of ES cell suspension was transferred over the wells of a microwell petri dish (Genome Systems Inc., MO). (Microwell plates can be prepared by ordinary tools available in the lab as described earlier[15]). Approximately ten minutes later, 10-15 cells settled down in each microwell. The zona-free embryos were then transferred over the ES cells, one embryo in each microwell and the embryos were allowed to culture overnight at 37°C in a CO_2 incubator.

Transfer of Embryos into Pseudopregnant Mothers

After overnight culture, the blastocysts were collected and washed in 3-5 ml of medium A. Approximately ten embryos were surgically transferred into one uterine horn of 2.5-day pseudopregnant CD1 female mice purchased from Charles River Laboratories (Wilmington, MA). The pups were born after about 17 days of transfer. CD1 females are generally good foster mothers for the newborn pups of manipulated embryos. At 2-3 weeks of age, the pups with high contributions of ES cells were separated. Chimeric males at the about eight weeks old were crossed with wild-type C57BL/6 females to obtain germline transmission of the *neo* gene. The progeny was analyzed by PCR of tail DNA to identify transgenic animals. Eight-week-old transgenic males were crossed with normal females to analyze expression of the transgene in progeny.

Tissue Culture Analysis of the Transgene

To investigate whether the transgenic animals express the *neo* gene, a transgenic male from the above cross was mated with a wild-type female. Five days after birth, transgenic pups and normal littermates were sacrificed to remove their tails and skin. The tissue was minced and treated with 1 ml of 0.25% trypsin EDTA for ten minutes at 37°C in a CO_2 incubator. The cells were pipetted several times to break tissue aggregates, and allowed to incubate for ten minutes followed by the addition of 15 ml of complete medium (Dulbecco's modified Eagle medium, DMEM, with 10% fetal calf serum). The cells were then transferred to a 100 mm tissue culture plate. After about 48 hours, plates were washed thoroughly to remove tissue pieces. At confluency, the cells were harvested by treating with trypsin EDTA, and transferred to three 60 mm petri dishes at a concentration of 1 x 10^5 cells each. After about four days, the cells were treated with 300 µg and 500 µg/ml G418. The medium was changed everyday to remove cell debris.

Results

Electroporation of DNA and Selection of ES Cell Clones

About 5 x 10^6 ES cells were electroporated with the purified DNA for the *neo* gene (Fig. 1), and the cells were transferred into six 100 mm petri dishes. After 48 hours, five plates were treated with 500 µg/ml G418. As a control, one petri dish was treated with 150 µg/ml G418, a drug concentration usually used for screening gene knockout clones[24]. After 7-8 days of G418 treatment, only about half the colonies survived at 500 µg/ml concentration, compared to those at 150 µg/ml G418 concentration (1,700 and 3,200 colonies, respectively) suggesting that colonies with integration sites with low expression of the *neo* gene were eliminated. About 50 independent colonies were collected from the petri dishes with 500 µg/ml G418.

Southern Analysis of ES Cells

Each colony of ES cells was trypsinized individually, and transferred each to a 24-well plate with PEF feeder cells. After 48 hours, the cells were harvested by trypsinization and an aliquot of the cells was cultured on a petri dish without feeder cells. The remaining cells were stored in liquid nitrogen.

After 5-7 days of culture, the cells were lysed to isolate DNA[3]. Using BamHI restriction enzyme, 10 µg of DNA were digested and analyzed by Southern blot analysis using p32-labeled *neo* specific probe. The modified *neo* gene contains only one restriction site for BamHI enzyme[20], therefore only two bands of unique size comprising *neo* sequences along with the flanking 5′- and 3′-sequences from the host genome are expected from single-copy integration. However, it is possible that the gene may integrate as a head-to-tail multimeric unit[1]; in that situation, in addition to unique bands representing flanking sequences, an internal band of size equal to the transgene (1.13 kb) will also be generated. The intensity of this band will depend on the number of copies of the integrated DNA. A second possibility is

FIGURE 3. ES cells from clone 1 were cultured with the C57BL/6 morulas[19]. Three male mice displayed almost complete agouti coat color compared to the wild-type black animal shown.

that the DNA may integrate at more than one site; as a consequence, each independent site will generate its unique bands. The integrated DNA may also undergo rearrangement at certain sites that will lead to a complicated pattern of bands.

Fig. 2 shows Southern blot analysis of four independent ES clones. Clones 2, 3, and 4 showed multiple bands of size between approximately 2.2 kb and 12.0 kb, and we observed no band equal to the size of the transgene, which suggests that the clones may have integration occurring at more than one site. Clone 1, however, displayed only two unique bands of approximately 13.0 kb and 8.0 kb with almost equal intensity, which suggests a single copy integration of the gene.

Preparation of Chimeric Animals

ES cells from clone 1 were co-cultured with zona-free morulas as described in the literature[15]. A total of 140 embryos were transferred into pseudopregnant females, producing 48 pups. Thirty-four pups survived; 14 pups were either cannibalized, or died from unknown causes. R1 ES cells originate from the 129Sv strain of mice with agouti coat color. Therefore, the chimeric animals are expected to display patches of agouti color over the host embryo's black background. Based on visual inspection, 14 animals (approximately 41%) displayed 40-100% ES cell contribution (Table 1). Two pups were approximately 80% chimeric (not shown), whereas three animals displayed almost pure agouti coat color suggesting high ES cell contribution (Fig. 3). Although low chimeric animals are less efficient in transmitting the gene to their progeny, the overall efficiency of chimeric production by this method is comparable to 10-20% of transgenic pups by DNA microinjection[21].

Germline Transmission of the Transgene

All the chimeric mice appeared healthy and normal. To investigate whether they transmit the gene of interest to their progeny, one of the males with a high contribution of ES cells was crossed with wild-type C57BL/6 females, and the progeny were analyzed by PCR. The DNA isolated from the toes of the pups amplified an expected band of 330 bp (data not shown) confirming that ES cells participated in the formation of the germline. To check whether the transgenic pups from chimeric mice also transmit the gene of interest to their progeny, a 7-8 week-old transgenic male was crossed with a wild-type C57BL/6 female to obtain F1 progeny. A total of eight pups were born from this mating. Five days after birth, the pups were sacrificed; a small piece of tail was excised to analyze for neomycin gene, and the remaining tissues were used to establish cell lines for the expression of the *neo* gene. PCR analysis revealed that four pups amplified the *neo* specific 330 bp band (Fig. 4; lanes 1, 2, 5, and 6) confirming that the transgenic animals transmitted the gene to their progeny in normal Mendelian fashion.

Expression of Gene in Transgenic Animals

After PCR analysis, tail and skin of F1 transgenic and normal pups were minced and cultured to establish fibroblast cells. Approximately 1 x 10^5 cells were cultured in 60 mm petri dishes. After four days, the cells were treated with different concentrations of G418. As shown in Fig. 5, the cells from transgenic mice survived at both concentrations of G418. There were about 20% more cells in the petri dish treated with 300 µg/ml G418 (Fig. 5E) compared to 500 µg/ml concentration (Fig. 5F). Overall, the cells appeared healthy and normal. On the other hand, 90% of the cells from nontransgenic littermates died within 24-48 hours of treatment (not shown), and almost all the cells died after 6-8 days of treatment (Figs. 5B and 5C). There was no apparent difference between the untreated cells from transgenic and wild-type littermates (Figs. 5D and 5A, respectively).

Summary of Steps to Prepare Transgenic Animals by Morula Aggregation

The following steps summarize the preparation of transgenic animals by morula aggregation with pre-selected ES cells:

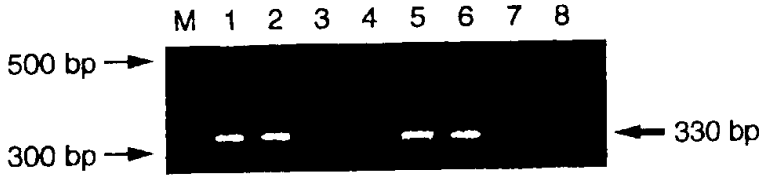


FIGURE 4. Polymerase chain reaction (PCR) analysis of the progeny of transgenic animal is shown. Tails from eight pups born from a cross between transgenic male and a wild-type female were analyzed by PCR. DNA from pups 1, 2, 5, and 6 amplified an expected band of 330 bp, confirming the germline transmission of the neomycin gene.

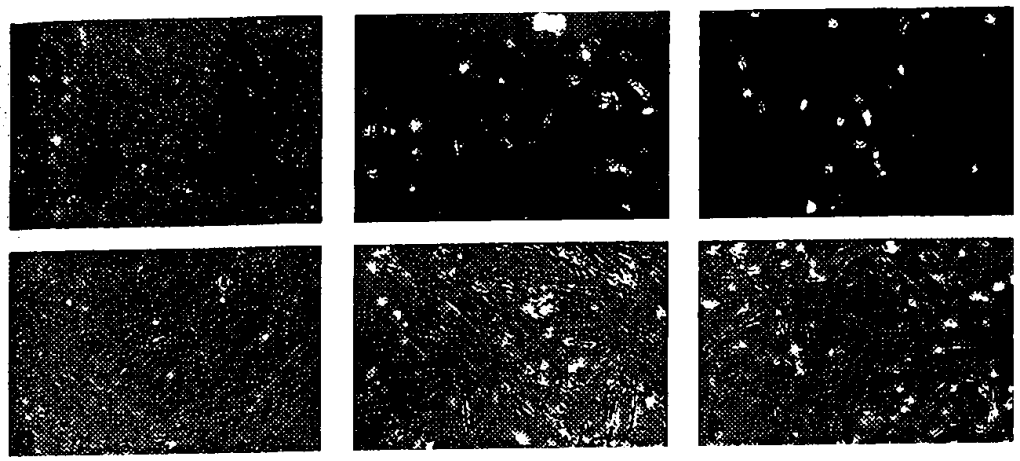


FIGURE 5. Tissue culture of cells from the skin and tails of five-day-old wild-type (A, B, C) and transgenic (D, E, F) littermates were cultured. After 4-5 days, the cells were treated with 300 µg/ml (B, E) and 500 µg/ml (C, F) G418. The cells from wild-type animals (B, C) died after G418 treatment whereas cells from transgenic animals were resistant to such treatment (E, F). There was no apparent difference between untreated cells from wild-type (A) and transgenic (D) animals. Magnification 60x.

1. Electroporation of DNA containing selectable gene into ES cells;
2. Selection of clones using a high concentration (500 µg/ml or more) of G418;
3. Screening of clones for single-copy DNA by Southern blot analysis;
4. Aggregation of ES cells with zona-free morulas;
5. Transfer of embryos to pseudopregnant females to obtain chimeric pups;
6. Mating of chimeras with wild-type animals for germline transmission;
7. Mating of transgenic progeny to establish lines;
8. Analysis of animals for phenotype and gene expression.

Discussion

The microinjection of DNA into fertilized embryos is a powerful tool for preparing genetically modified animals[1,22]. The DNA generally integrates into the host genome and gets transmitted to the subsequent generations. The procedure, however, is tedious and requires highly specialized skills and expensive equipment, which limit its application by most laboratories. The other limitations are that the expression of integrated DNA cannot be predicted, which can be frustrating since the results are known only after several months. The gene integration may occur at a site where the expression is too low. As a result, the animal may not exhibit any phenotype. Further, the integration of tandemly arranged copies may lead to variegated expression of the gene[23], which may complicate the interpretation of results. All these factors contribute to the loss of a tremendous amount of time and effort. A simple system which alleviates these problems can greatly expedite and help predict the outcome of transgenic experiments.

ES cells have been used extensively to prepare gene knockout animals via homologous recombination[7-11,20,24], and may be used to prepare animals with random integration of DNA[1]. ES cells offer several advantages over DNA microinjection. First, integrants for single gene copy can be predetermined before preparing chimeric animals. Second, including a selectable marker such as the *neo* gene in the same construct can identify the integration sites for gene expression. Since the gene of interest and the selectable gene will be integrated at the same site, it is likely that the gene of interest will also be expressed as directed by the spatial and temporal specificity of its own promoter. Third, the cells with integrants for potentially high expression can be selected by applying higher dosage of the drug. Fourth and most important, transgenic animals can be prepared conveniently without the need for expensive equipment.

Recently, ES cells pre-selected for green fluorescent protein (GFP) expression were used to prepare transgenic mice[25]. The neomycin gene used in the current studies offers the advantage over other markers because high doses of G418 can be used to select high expressing clones. Our data showed that approximately 50% fewer colonies were obtained at 500 µg/ml G418 (versus 150 µg/ml), suggesting that colonies with low expression of *neo* gene died at high dosages of drug. Further, with higher dosage (2 mg/ml or higher), it is possible to select clones homozygous for the transgene[26], which will enhance the chances of germline transmission.

Neo gene is relatively small and may not test the size limitations of the commonly used vectors. Further, its expression is non-toxic to the ES cells. In some cases, the selector gene has caused some unexpected consequences[27]; to alleviate such problems, a common gene cassette may be designed in which the selector gene is flanked by bacteriophage P1 lox sequences[28,29]. After G418 selection, the cells can be transfected with a construct containing the gene for *cre*-recombinase driven by a promoter that expresses in ES cells to excise the selector gene. Alternatively, several independent ES clones may be mixed to prepare a compound transgenic animal, which will transmit different integrants independent of each other. Transgenic mice with desired characteristics may be then propagated to establish lines.

The data presented here demonstrate that ES cells can be a convenient alternative to DNA microinjection when preparing transgenic animals. The efficiency of generating chimeric pups by morula aggregation (41%) is quite comparable to ES cell injection (38%) into blastocysts[10]. Pre-selection of ES cells offers tremendous advantages for preparing transgenic animals with high gene expression. The

approach may be of particular advantage for larger species, in which one-cell embryos are difficult to obtain and gestation periods are usually long.

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